The University of Chicago

Bacterial Spores and the Development of Flagella on Newly Germinated Spores

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF HYGIENE AND BACTERIOLOGY

BY

EINAR LEIFSON

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BACTERIAL SPORES[1]

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INTRODUCTION

According to Teasi Matzuschita (1902) bacterial spores were first recognized and described by Pertz in 1852 in a book entitled "Zur Kenntniss kleinster Lebensformen." Since the discovery of *B. anthracis* the nature of bacterial spores and the conditions under which they are formed have been extensively studied. A number of organisms, including *Bact. typhosum* and *M. tuberculosis*, were for a time believed to form spores. This has now definitely been disproved. Heat resistance of a culture has become the generally accepted criterion of the presence of spores. Spores generally have a thermal death point 30° or 40°C. higher than that of vegetative cells.

It seems quite definitely proved that sporulation may occur in any spore forming bacteria irrespective of the number of generations they are removed from the parent spores. According to Garbowsky (1907) the very first organisms which developed from a spore are capable of sporulating. Schreiber (1896) has substantiated this observation. Concerning pre-spore changes which take place in the organisms there seem to be considerable differences of opinion. Many of the early investigators believed that the spore resulted from a condensation of large pre-spore granules. Subsequent work, however, especially by Schaudinn (1902), Guilliermond (1910), and Schwellengrebel (1913), shows that

[1] Part of a dissertation submitted to the Department of Hygiene and Bacteriology of the University of Chicago to fulfil certain requirements for the degree of Doctor of Philosophy.

such granulation does not always take place. These investigators find that at the time of sporulation chromatin granules appear in the cells. These granules arrange themselves in certain patterns and together with other constituents of the cell condense to form the spore. A great deal has been written about the presence of nucleii in spore forming bacteria and their relation to sporulation. No definite agreement or conclusions seem to have been reached, however. Bunge (1895), and Grethe (1897) first expressed the idea that the proteins of the vegetative cells and of the spores are different. Later workers, including Ruzicka (1913), and Mellon and Anderson (1919) have expressed the same idea. Chemical analysis of spores seem to show that they consist mostly of proteins. Cramer (1894) found the protein content to be 70 to 90 per cent

Extensive investigations have been made of the properties of spores, and especially their resistance to heat and chemicals. The conditions under which they are stored seem to have an effect on their resistance to heat and chemicals. Some of the later investigations on the effect of heat are those of Burke (1923), Bigelow (1921), Reiter (1920), Esty and Meyer (1922), Magoon (1926), and Williams (1929). It seems definitely established that certain media produce spores of higher thermal resistance than other media. As to the effect of age and conditions of storage there seems to be a lack of agreement. The effect of chemicals has been extensively studied, especially by Müller (1920) and Dozier (1924). It seems that under certain conditions chemicals which inhibit sporulation lead to the production of asporogenic strains of the organisms. *B. anthracis* seems to form asporogenic strains most readily.

The conditions most favorable for sporulation have been found to be essentially those most favorable for growth, except for the absence of food (Buchner (1890), Weil (1899), Matzuschita (1902)). The effect of oxygen has been extensively studied by Meyer (1909) and Bredeman (1909). In general, the optimum oxygen concentration for the sporulation of aerobes seems to be atmospheric concentration, and for that of anaerobes close to 0. The optimum temperature for sporulation corresponds closely

with that for growth. The limiting temperature for the sporulation of most of the ordinary bacteria is between 15° and 45°C. A good review and discussion of this subject is given by Holzmüller (1909).

OBSERVATIONS FROM EXPERIMENT

Hydrogen-ion concentration and sporulation

The hydrogen-ion concentration of a medium has a considerable influence on sporulation. The idea seems to have become general that an alkaline medium is more favorable for sporulation than an acid one. The experience of the author has been that a neutral or slightly acid medium is more favorable for sporulation than an

TABLE 1

Sporulation at different hydrogen-ion concentrations

MEDIUM NUMBER	Na_2HPO_4 (0.3M)	NaH_2PO_4 (0.3M)	pH FINAL	SPORES			
				Cl. botulinum	*Cl. tetani*	*B. vulgatus*	*B. circulans*
	cc.	*cc.*		*per cent*	*per cent*	*per cent*	*per cent*
1	0	1.0	5.1	No growth	2	80	No growth
2	0.05	0.95	5.6	No growth	10	90	?
3	0.15	0.85	6.0	90	40	90	<1
4	0.20	0.80	6.2	90	80	90	10
5	0.30	0.70	6.4	50	40	90	5
6	0.90	0.1	8	<1	5	20	<1
7	0.95	0.05	8.4	<1	2	40	<1

alkaline one. It is not so much the initial pH of the medium (nor the final one) which is important but the pH at the time of sporulation.

A number of experiments were performed to determine the optimum hydrogen-ion concentration for the sporulation of *Cl. botulinum, Cl. tetani, B. vulgatus,* and *B. circulans.* More experiments were made with *Cl. botulinum* than with the others. The medium used was 2 per cent agar in slants with 1 per cent peptone, 0.5 per cent beef extract, and the buffer mixtures as indicated. The cultures were all incubated at 37°C. and the anaerobes in an anaerobic jar. A typical result is given in table 1.

It is concluded from these experiments that for *Cl. botulinum*, *Cl. tetani*, *B. vulgatus*, and *B. circulans* the optimum hydrogen-ion concentration for sporulation on phosphate buffered extract agar is distinctly acid and at a pH of about 6.2.

Effect of carbohydrates on the sporulation of Cl. botulinum

The presence of a fermentable sugar in a medium generally inhibits sporulation. The cause of this inhibition may be (1) the acid produced, (2) the carbon dioxide produced, or (3) the carbohydrate as such. Non-fermentable sugars have no inhibiting effect on sporulation. *Cl. botulinum*, for example, will grow and sporulate in 60 per cent sucrose.

TABLE 2

Effect of various concentrations of glucose on the sporulation of Cl. botulinum

MEDIUM NUMBER	GLUCOSE	pH		pH CHANGE	SPORES
		Initial	Final		
	per cent				
1	1	6.87	5.35	−1.48	−
2	0.8	—	5.53	—	−
3	0.6	6.98	5.61	−1.37	−
4	0.4	7.00	5.93	−1.07	−
5	0.2	7.00	6.49	−0.51	+
6	0.1	7.00	6.55	−0.45	++
7	0	7.00	6.85	−0.15	++

A number of experiments were performed to determine the effect of glucose on the sporulation of *Cl. botulinum*. To a basic medium of extract bouillon were added varying amounts of glucose. The results after anaerobic incubation at 37° for four days are given in table 2.

It would seem from table 2 that whenever sufficient acid is produced to bring the pH of the medium below a certain value no sporulation occurs.

The limiting hydrogen-ion concentration for the sporulation of *Cl. botulinum* was previously found to be about pH 6.0. This would tend to indicate that the acid produced by the fermentation of the sugar is responsible for the inhibition of sporulation.

TABLE 3

Effect of various salts on growth and sporulation of Cl. botulinum and Cl. tetani

SALT	CONCENTRATION	pH INITIAL	CL. BOTULINUM		CL. TETANI	
			Growth	Spores	Growth	Spores
	per cent					
Na_2HPO_4	4	7.9	+	+	+	+
	8		+	+	+	+
	12		+	−	+	+
	16		+	−	+	+
	20		−		−	
Na_2SO_4	2	7.8	++	−	+	+
	4		++	+	+	+
	6		++	+	+	+
	8		++	+	+	+
	10		+	A few	+	+
NaCl	3	7.6	++	−	++	++
	6	7.7	+	−	−	
	9		−		−	
Na-citrate	4	7.8	+	+	−	
	8		−			
Na-oxalate	0.4	7.7	+	A few	+	+
	0.8	7.9	+	+	+	+
	1.2		+	+	+	+
	1.6	8.1	+	−	−	
	2.0		−		−	
K-phthalate	2	7.8	+	−	+	++
	4		+	++	+	−
	6		+	+	+	−
	8		+	−	Poor	−
	10	8.1	+	−	?	−
K_2SO_4	2	7.8	+	+	+	+
	4		+	++	+	+
	6		+	+	+	+
	8		+	A few	+	+
	10		Poor	−	+	A few
KCl	3	7.5	+	−	+	++
	6		Poor	−	+	−
	9		−		Poor	−
	12				−	

TABLE 3—*Continued*

SALT	CONCENTRATION	pH INITIAL	CL. BOTULINUM		CL. TETANI	
			Growth	Spores	Growth	Spores
	per cent					
$MgSO_4$	2.4		+	A few	+	+
	4.8		+	A few	+	++
	7.2	7.4	−		+	+
	9.6				+	+
	12				+	Poor
$MgCl_2$	2		+	+	+	++
	4		Poor	−	+	+
	6	7.1	−		?	−
$Ca(NO_3)_2$	0.2		+	−	+	+
	1		+	−	+	+
	3		+	−	+	+
	6	6.8	−		−	
$CaCl_2$	1	6.7	+	−	+	+
	2		−		+	−
	3				−	
Ca-lactate	2				+	+
	4	6.8	+	+	+	+
	6		+	+	+	+
	8		+	A few	+	+
	10		−		−	
$BaCl_2$	0.5	6.9	+	−	+	+
	1		−		+	−
	2				−	
Pb-acetate	0.008	7.2	+	−	+	+
	0.04		+	−	+	+
NH_4Cl	0.8	7.0	+	+	+	+++
	1.6		+	++	+	+++
	2.4		+	++	+	+++
	3.2	6.7	−		+	++
	4.0				−	
NH_4-phosphate	2	7.1	+	+++	+	+++
	4		+	++	−	
	6		−			

TABLE 3—*Concluded*

SALT	CONCENTRATION	pH INITIAL	CL. BOTULINUM		CL. TETANI	
			Growth	Spores	Growth	Spores
	per cent					
NH_4-citrate	1	7.4	+	+	+	++
	2	7.2	−		−	
NH_4-oxalate	0.5	7.4	+	+	+	++
	1	7.4	−		−	
NH_4-sulphate	2	6.9	+	+++	+	+++
	4		−		+	++
	6	6.8			−	
NH_4Br	1	7.3	+	++	+	++
	2		+	++	+	+++
	3		+	++	+	++
	4		+	+	+	+

Effect of inorganic salts on sporulation

The above table 3 gives the effects on sporulation of *Cl. botulinum* and *Cl. tetani* of a number of inorganic salts when added to a basic medium consisting of 1 per cent peptone, and 0.5 per cent beef extract. The tubes were incubated anaerobically for seven days. Table 3 shows that almost invariably growth occurs at a higher concentration than sporulation. Chlorides and nitrates prevent sporulation of *Cl. botulinum* but not to the same extent as in the case of *Cl. tetani*. Ammonium salts and sulphates stimulate sporulation of both organisms.

Chemical elements necessary for sporulation

Extract bouillon itself has all the ingredients necessary for some sporulation. The effect of added salts, whether stimulating or inhibiting, may be either chemical, physical, or both. If the salts are added to a basic medium consisting of peptone only, a better understanding of the effect of the salt may perhaps be obtained. One such experiment was performed. The composition of the media and the results, after five days of anaerobic incubation, are given in table 4.

The media in table 4 which show sporulation in both instances are nos. 3, 10, and 13. All three of these media contain both ammonium and phosphate ions. None of the other media except 15 have both of these ions. Medium 9 showed a few spores in the first set but not in the second. This medium contained ammo-

TABLE 4

Sporulation of Cl. botulinum in various media containing 1 per cent peptone plus various salts as indicated

MEDIA	SALTS ADDED	pH	FIRST SET		SECOND SET	
			Growth	Spores	Growth	Spores
				per cent		*per cent*
1		7.9	++	−	++	−
2	NH_4Cl−0.2M	7.3	+	−	+	−
3	$(NH_4)H_2PO_4$−0.2M	6.2	−		−	
	$(NH_4)_2HPO_4$−0.2M	7.2	++	10	++	5
4	$(NH_4)_2SO_4$−0.2M	6.4	−		+	
5	NaH_2PO_4−0.2M	6.0	−		−	−
	Na_2HPO_4−0.2M	7.9	++	−	++	−
	Na_3PO_4−0.2M	8.5	+	−	+	−
6	K_2SO_4−0.2M	8.1	+	−	+	−
7	$CaCl_2$−0.02M	7.8	++	−	++	−
8	$MgCl_2$−0.02M	8.0	++	−	++	−
9	NH_4Cl−0.2M + $CaCl_2$−0.02M	7.3	+	1	+	−
10	$(NH_4)H_2PO_4$−0.15M + $CaCl_2$−0.05M	6.1	−		−	
	$(NH_4)_2HPO_4$−0.15M + $CaCl_2$−0.05M	6.9	++	20	++	15
11	$(NH_4)_2SO_4$−0.15M + $MgCl_2$−0.05M	7.3	+	−	+	−
12	Na_2HPO_4−0.15M + $MgCl_2$−0.05M	7.7	++	−	++	−
13	$(NH_4)_2SO_4$−0.1M + Na_2HPO_4−0.1M + $MgCl_2$−0.05M	6.9	++	1	++	1
14	NaH_2PO_4−0.1M + K_2SO_4−0.1M	6.6	++	−	++	−
	Na_2HPO_4−0.1M + K_2SO_4−0.1M	7.1	++	−	++	−
	Na_3PO_4−0.1M + K_2SO_4−0.1M	8.4	+	−	+	−
15	$(NH_4)_2HPO_4$−0.15M + $MgCl_2$−0.05M	6.9	+	−	+	−

nium and calcium ions. It seems that ammonium and phosphate ions are essential for the sporulation of *Cl. botulinum*. Beef extract may be somewhat deficient in these ions and hence the stimulating effect on sporulation when they are added to extract bouillon. The calcium ion also seems to have some stimulating effect.

Effect of oxygen on sporulation

Sporulating organisms seem to be either strict aerobes or strict anaerobes. The optimum concentration of oxygen for the sporulation of aerobes has been extensively studied in the past by Meyer (1909) and others. In the case of strict anaerobes the optimum oxygen concentration for sporulation is very close to

TABLE 5

Effect of oxygen on the sporulation of a number of aerobes and anaerobes

ORGANISMS	OXYGEN TENSION OF MERCURY					
	0 cm.*		1 cm.		2 cm.	
	Growth	Spores	Growth	Spores	Growth	Spores
		per cent		*per cent*		
Cl. botulinum A	+++	95	+	10	0	
Cl. botulinum B	+++	80	+	10	0	
Cl. chauveii	+++	90	+	90	+	0
Cl. sporogenes	+++	90	+	90	+	0
Cl. tetani (atypical)	+++	95	+	50	0	
Cl. tetani	++	30	+	0	0	
Cl. novyi	+	2	0		0	
Cl. oedematiens	+++	50	+	60	0	
Cl. septique	+++	95	+	40	+	0
B. anthracis	0		0		++	0
B. vulgatus	0		1 colony?	30	+++	?
B. circulans	+	0	++	0	+++	0
B. cereus	0		0		+	0

* This oxygen tension was undoubtedly greater than zero. Just what it was is impossible to say. The agar was freshly boiled. The nitrogen was passed through a long column of yellow phosphorus before it was admitted to the anaerobic jar. The water in the bottom of the jar was allowed to boil under the vacuum for some time. Any oxygen left must certainly have been at a very low concentration.

zero. Table 5 gives the results of a number of experiments on this subject. The organisms were grown on agar slants in anaerobic jars which had first been freed of oxygen by alternately evacuating and filling with nitrogen and then the desired amount of air let in as measured by a manometer. The aerobes and anaerobes were kept in separate jars. The cultures were incubated for four days.

It may be observed from table 5 that the anaerobes show a considerable difference in their ability to sporulate under increased oxygen tensions. The sporulation of *Cl. tetani* and *Cl. novyi* was inhibited at 1 cm. of oxygen. All the others except *Cl. sporogenes* and *Cl. chauveii* sporulated less at 1 cm. of oxygen than at 0 cm. (see note). The sporulation of all the organisms was inhibited by 2 cm. of oxygen. In the case of the aerobes no sporulation was observed at 2 cm. of oxygen although fair growth was obtained. *B. circulans* seems to be the most "anaerobic" of the aerobes used —growing as it did in less than 1 cm. of oxygen.

Most strict aerobes sporulate poorly in bouillon, mainly because of the low oxygen concentration. It has been my practice to place a freshly inoculated slant of *B. subtilis* or *B. anthracis* in my anaerobic jars as a check on the anaerobic condition. If the aerobe does not grow, the air tension is probably below 1 cm. of mercury as indicated by my own work and that of others. If it grows but does not sporulate, the air tension is probably between 1 and 4 cm. of mercury.

Experiments with Cl. botulinum

The changes which take place in the properties of an organism as it passes through its spore cycle have ordinarily been studied separately. In the following pages are presented the results of some experiments with *Cl. botulinum* type A, in which the change of a number of properties was studied simultaneously as the organism passed through its spore cycle. The following changes of the organisms and the medium were determined: General morphology of the organisms, pH of the medium, cataphoretic potential of the organisms, oxygen sensitivity of the organisms, thermal death time-temperature of the organisms, and toxicity of the medium.

Culture medium

The experiments, first of all, demanded a medium in which the organisms could be made to germinate and sporulate within definite time limits, and in which the sporulation would be at least 90 per cent. Such a medium had been developed earlier in

the work. The medium consists of 1 per cent peptone (Wilson), 0.5 per cent beef extract (Swift), 0.5 per cent Na_3PO_4, + 0.5 per cent $(NH_4)_2SO_4$. No adjustment of reaction was made, and it was found to vary from pH 6.7 to pH 6.9. For the nutrient agar, 2 per cent agar was added. In this medium germination occurred in from fifteen to twenty hours and sporulation in from forty to forty-eight hours. The degree of sporulation varied from 90 to 100 per cent.

Anaerobic jars

The next requirement was a number of anaerobic jars which could be handled with ease, and which would allow regular growth on agar slants. A very convenient and inexpensive anaerobic jar was made as follows: Into empty peptone jars (or similar glass jars) were placed two-hole rubber stoppers. Into one hole was placed a mercury manometer and into the other a two-way stopcock, as shown in figure 1. In the bottom of each jar was placed a small quantity of pyrogallic acid (3 to 5 grams), and a few grams of Na_2CO_3. Na_2CO_3 was used in place of NaOH because of the latter's affinity for CO_2. The presence of a strong solution of NaOH was found to inhibit growth on agar slants. The rubber stopper was greased with a mixture of vaseline and beeswax, and the stopcock with a heavy rubber grease. At incubator temperature (37°C.) ordinary vaseline and stopcock grease are too liquid to be efficient. When the tubes were in the jar a little water was added by means of a pipette, and the jar evacuated with a water pump. Connection with a nitrogen tank was made by one arm of the stopcock. As the vacuum was being built up a little nitrogen was run in to remove the air in the arm of the stopcock. The jar could then be filled with pure nitrogen after evacuation by the water pump. To facilitate removal of the oxygen the water in the bottom of the jar was kept warm so that it boiled under the vacuum. The nitrogen was freed of oxygen by passing it through a long column of yellow phosphorus, followed by several bottles of alkali to remove the P_2O_5 formed. Commercial nitrogen seems to contain a considerable amount of oxygen and is

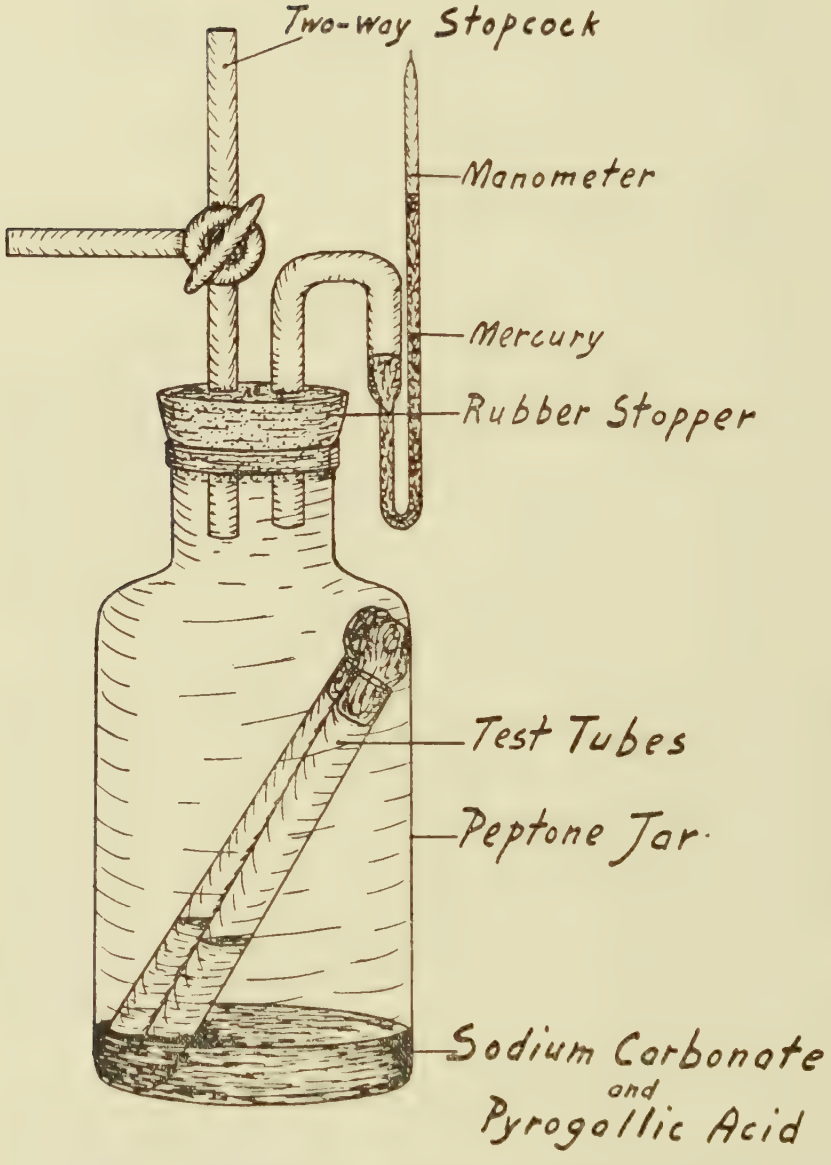

FIG. 1. A SIMPLE AND INEXPENSIVE ANAEROBIC JAR

not to be recommended for use in anaerobic work. Commercial hydrogen is much purer.

Method of conducting the experiment

A number of freshly slanted agar slants were inoculated with *Cl. botulinum* type A, and incubated at 37°C. for four days. The jar was then opened and the slants left in the incubator for one day. This exposure to air at 37°C. was found to disintegrate completely the vegetative part of the cells in which spores had formed. The spores were then removed from the slants, washed by centrifuging, suspended in bouillon, and heated at 70°C. for ten minutes to destroy any toxin they might contain. About 30 freshly boiled tubes of bouillon were inoculated at 50°C. from the spore suspension by means of a pipette. Four tubes of bouillon were placed in each of a number of anaerobic jars and incubated at 37°C. At different intervals of time, so chosen that the organisms would be in distinctly different developmental stages, the different jars were opened and the examination made. As soon as a jar was opened the tubes were placed in ice water to prevent further change.

Changes in morphology of the organisms

In the germination of *Cl. botulinum* the new vegetative cell comes out of the end of the spore opposite that to which the old vegetative cell is or was attached. The first sign of germination is swelling and increased affinity for stain. The vegetative cell then grows out of one end of the swelled spore. A great number of spores always fail to germinate. Germination was generally complete in from fifteen to twenty hours. In thirty-five to forty hours signs of sporulation began to appear as highly stainable swellings close to the end of the organism. These swellings gradually lost their affinity for stain and sporulation was complete in forty to forty-eight hours. The swollen spore preceding germination, the very first bud of vegetative cell coming out of the spore, and the swelling of the young spore were all intensely Gram positive.

Changes in pH of the medium

The pH determinations were made electrometrically by means of the quinhydrone electrode. The electrode vessel was made by cutting off two test tubes 2 inches from the bottom and sealing a stopcock between them. The stopcock formed a bridge between the two tubes. The whole apparatus was filled with the liquid to be tested. Quinhydrone and a gold electrode were placed in one tube

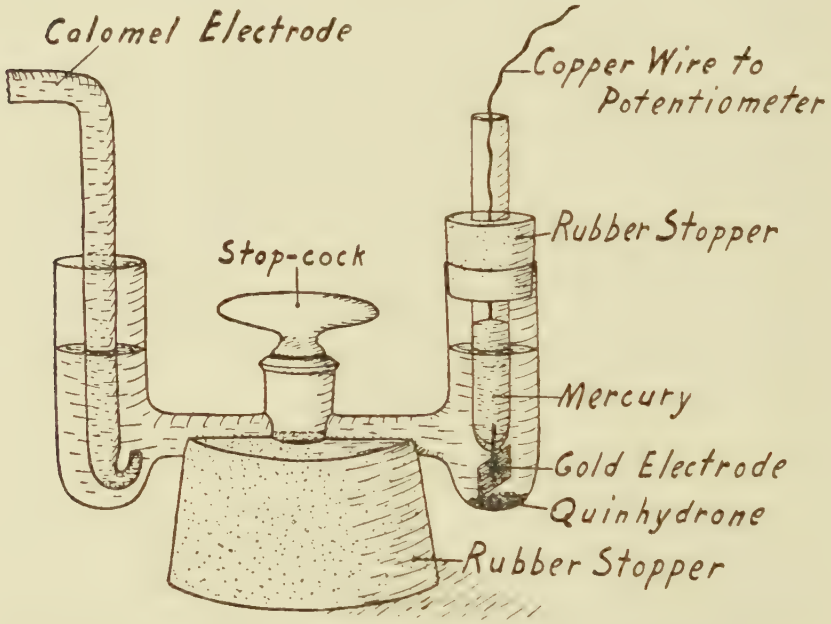

FIG. 2. APPARATUS FOR DETERMINING HYDROGEN-ION CONCENTRATIONS ELECTROMETRICALLY BY MEANS OF THE QUINHYDRONE ELECTRODE

of the apparatus, and the arm of a calomel cell in the other. A drawing of the apparatus is shown in figure 2. From three of the spore cycles a pH reading was taken from every jar. These three readings were averaged and the resulting curve is shown in figure 3. A change of pH of about 0.2 is noted at the time of sporulation. In a medium as well buffered as this one, the change is of some significance.

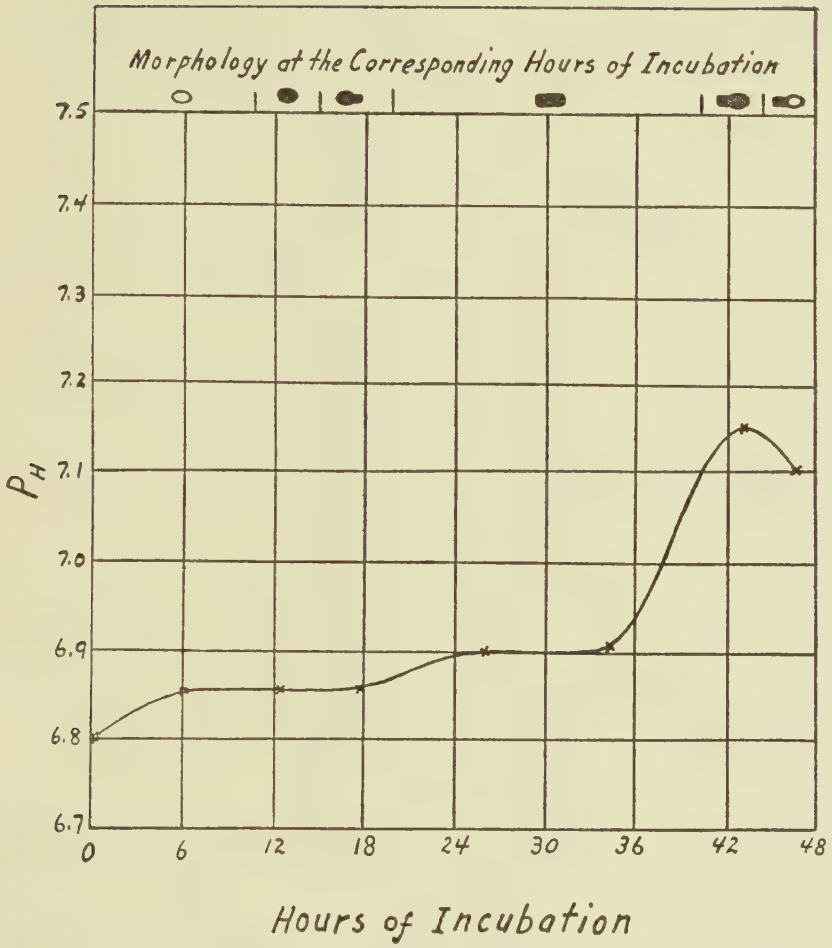

FIG. 3. AVERAGE HYDROGEN-ION CONCENTRATIONS OF THE CL. BOTULINUM CULTURES AFTER VARIOUS PERIODS OF INCUBATION

Changes in cataphoretic potential of the organisms

The data for this experiment were obtained by means of an apparatus with non-polarizable electrodes, somewhat similar to that originally described by Northrop. No attempt was made to standardize it since only relative values were desired. In each case the organisms were centrifuged and resuspended in the origi-

nal bouillon. The readings on the young vegetative cells are poor because of their motility. The readings of the different cycles are averaged and plotted in figure 4. The migration velocity is

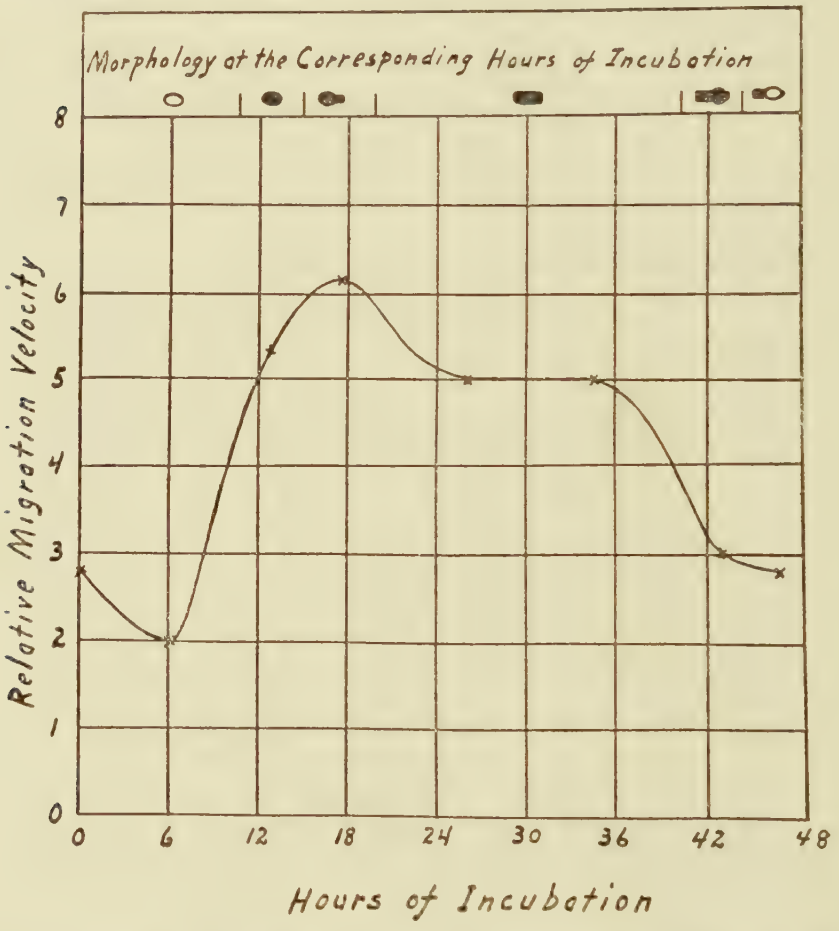

Fig. 4. Average Cataphoretic Potential of Cl. botulinum after Various Periods of Incubation

seen to decrease at first and then increase to a maximum, followed by a decrease to about that of the original spores. Whether the change in the cataphoretic potential indicates an actual change in

the nature of the cell proteins, the accumulation of non-diffusible by-products, or some other factor is impossible to say from the data at hand.

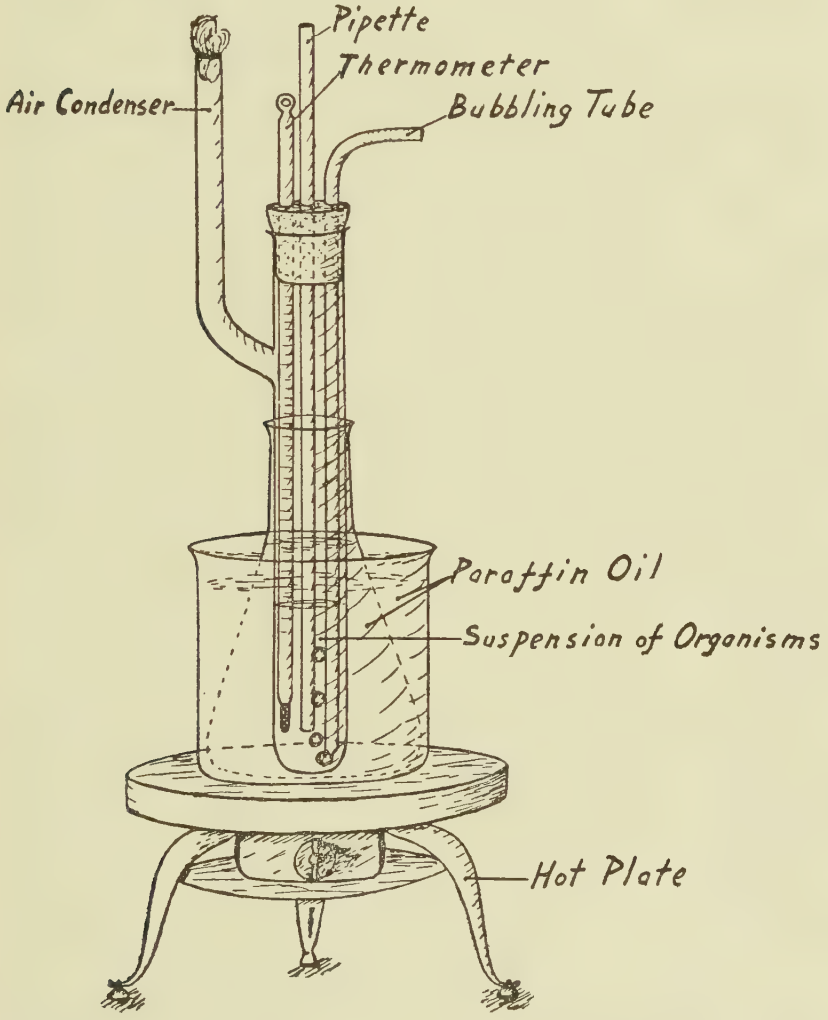

FIG. 5. APPARATUS FOR DETERMINING THE THERMAL DEATH TIME-TEMPERATURE OF CL. BOTULINUM AT VARIOUS PERIODS OF THE SPORE CYCLE

Changes of thermal death time-temperature

A determination of the thermal sensitivity of an organism in the process of germination or sporulation can obviously not be made in the ordinary way. The method adopted was to raise the temperature of the suspension of organisms gradually, and remove samples to agar shakes at 30°, 60°, 70°, 80°, and 90°C. respectively. The time was also taken. The apparatus consisted of a test tube fitted with an air condenser, and a three-hole rubber stopper. Through the holes in the stopper was put a thermometer, a capillary pipette for withdrawing samples, and a tube from a nitrogen tank. Oxygen-free nitrogen was kept bubbling through the suspension continuously to keep air out, and stir the suspension. A drawing of the apparatus is shown in figure 5. The time for the temperature to rise from about 25° to 60°C. was about twenty minutes, from 60° to 70°C. about six minutes, from 70° to 80°C. about seven minutes, and from 80° to 90°C. about eight minutes. The data show a gradual increase of thermal sensitivity as the organism germinates, and a decrease as it sporulates. It is interesting to note the low resistance of a majority of the original spores, only ten per cent surviving up to 90°C. The average thermal death point of most of these spores would then be between 80° and 90°C. The newly formed spores were even less resistant; only 1 to 2 per cent survived up to 90°C., 10 per cent up to 80°C., 20 per cent up to 70°C. and 50 per cent up to 60°C. The preliminary heating of the original spores to 70°C. for ten minutes accounts for the difference in heat resistance between these spores and those produced later. The observation is general that only a fraction of the spores formed in any culture are capable of germinating. This is especially true if the spores have been subjected to unfavorable conditions of any kind. Most spores are, therefore, probably failures as far as their chief function (?) is concerned of tiding the race of organisms over periods of unfavorable environmental conditions. It is doubtful if other media would have produced more heat resistant spores. An average of the different readings is plotted in figure 6.

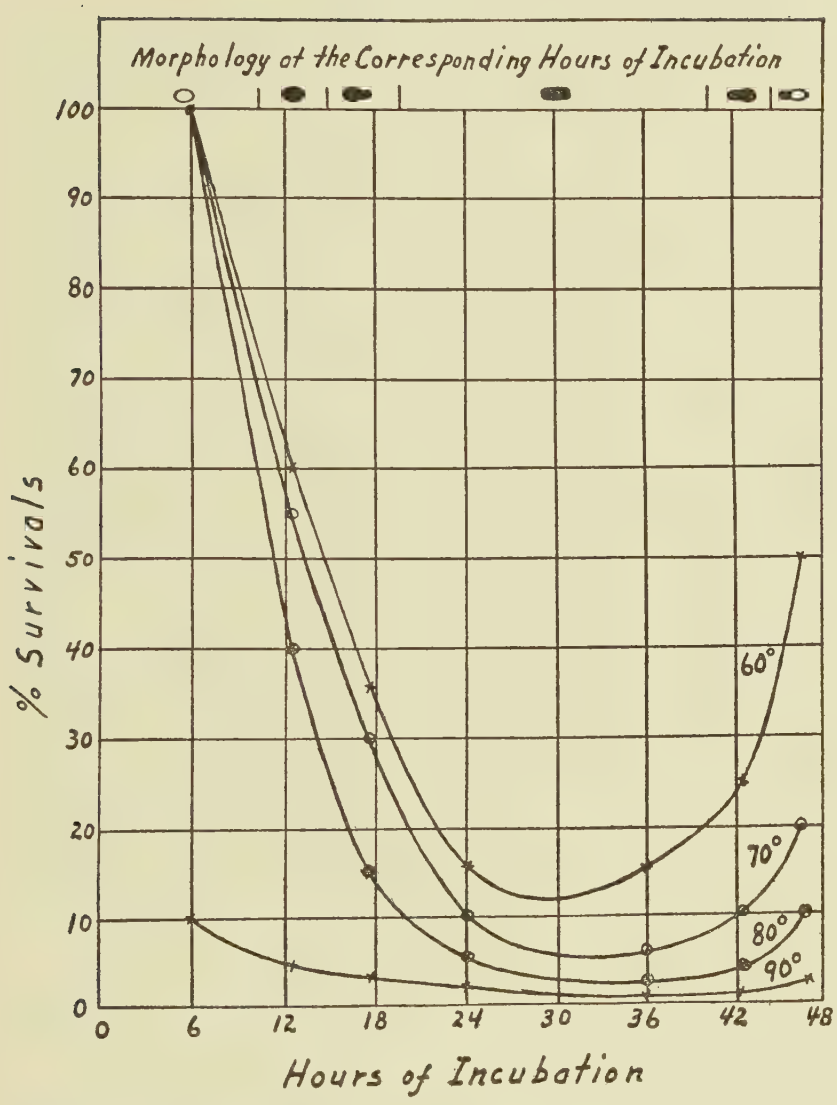

FIG. 6. AVERAGE PER CENT OF CL. BOTULINUM IN VARIOUS GROWTH PHASES SURVIVING HEATING AT VARIOUS TEMPERATURES

Change of oxygen sensitivity

Some anaerobes in the vegetative state are quickly killed by exposure to air. Previous work on the subject gives results which differ considerably. The present experiment was undertaken to correlate the change in sensitivity of *Cl. botulinum* with change in morphology as it passed through the spore cycle. The method was to grow the organisms in bouillon and at different morphologic stages remove a tube from the anaerobic jar and streak a loopful of the culture over an agar slant. The agar slants were then left exposed to the air, upside down, for different lengths of time; they were incubated anaerobically, and the number of colonies which developed counted. In the first few experiments agar slants of about pH 6, pH 7, and pH 8 were used, and exposed at temperatures of 8° and 37°C. (ice box and incubator). In this manner the effect on sensitivity of both pH and temperature could be obtained simultaneously. No differences in sensitivity of the organisms exposed at the different H-ion concentrations were found, nor in those exposed at the different temperatures. The shortest exposure in most of these cases was five minutes and it is possible that if shorter exposures had been tried a differential effect might have been found. In the later experiments only one kind of agar slants was used, pH 6.8 to 7, and all were exposed at 37°C. To express the results concisely is most difficult. In only one case of a considerable number was there obtained what may be considered a differential effect of different lengths of exposure. In this case the organisms were close to, if not in, the beginning stages of sporulation. The results of this one case were as follows:

Length of exposure *minutes*	Survivals *per cent*
1	100
6	100
11	25
46	15
70	5

These results may have been accidental. The method of expressing the results in figure 7 was to estimate the number of survivals after exposure to air for one hundred minutes and for five minutes.

It seems that organisms which are at all sensitive are generally killed by exposure of two minutes or less (two minutes was the shortest time possible with the method used). It appears from

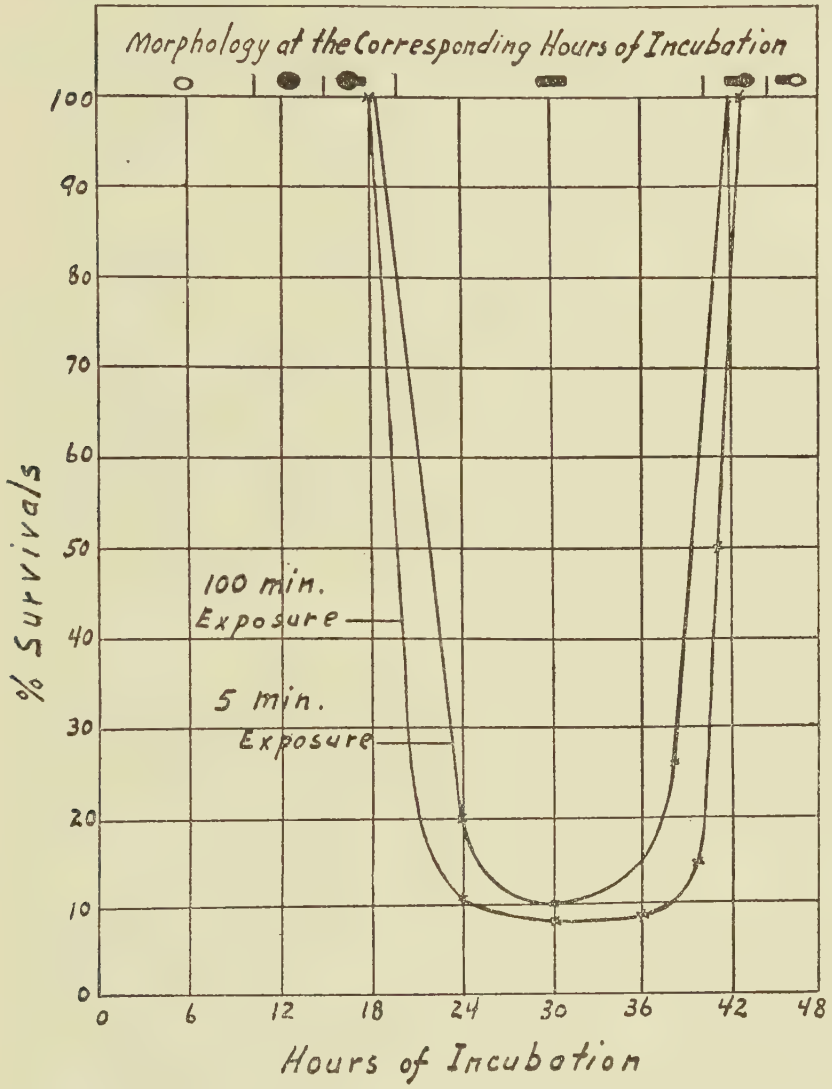

FIG. 7. AVERAGE PER CENT OF CL. BOTULINUM IN VARIOUS GROWTH PHASES SURVIVING EXPOSURE TO OXYGEN FOR FIVE AND ONE HUNDRED MINUTES

the curve that only vegetative cells are oxygen sensitive. Neither germinating spores nor sporulating vegetative cells show any appreciable sensitivity. The fact that the curves do not in any case reach the 0 line must not be taken to indicate that some vegetative cells are insensitive to oxygen but rather that there are present a number of ungerminated spores, which later germinate. It is possible that vegetative cells which do not sporulate lose their sensitivity as they become older. No data were obtained on this score.

Toxin production

The usual procedure in the production of botulinum toxin has been to cultivate the organisms for about a week. In a culture where at least 90 per cent of the organisms sporulate within forty-eight hours, interesting light on toxin production could perhaps be obtained. White mice were injected intraperitoneally with different quantities of supernatant broth after centrifuging out the organisms. In each case the total amount injected was $\frac{1}{2}$ cc. Antitoxin was injected to control the highest concentration injected. Every injection was made in duplicate. The three sets of data check very closely, and the average of the three is plotted in figure 8.

It is interesting to note that very little toxin is produced until the organism has reached the height of its growth stage. As sporulation begins, the toxin titer falls rather quickly and it seems as if no further toxin is produced. Upon exposure to air the toxin is destroyed very quickly. An experiment was made by mixing four tubes of toxic bouillon, and dividing the mixture into four tubes, care being taken to avoid exposure to air as much as possible. Two of these tubes were placed immediately under anaerobic conditions. The other two were thoroughly shaken in the air from time to time. The toxicity of the pooled broth was between 10,000 and 100,000 mice M.L.D. per cubic centimeter. After twenty-one hours the toxin titers were again tested. The anaerobic tubes gave a titer the same as the original. The exposed tube gave a titer of slightly over 100 M.L.D. After five

days the titer of the anaerobic tubes was somewhat over 100 M.L.D., and the aerobic tube somewhat over 10 M.L.D.

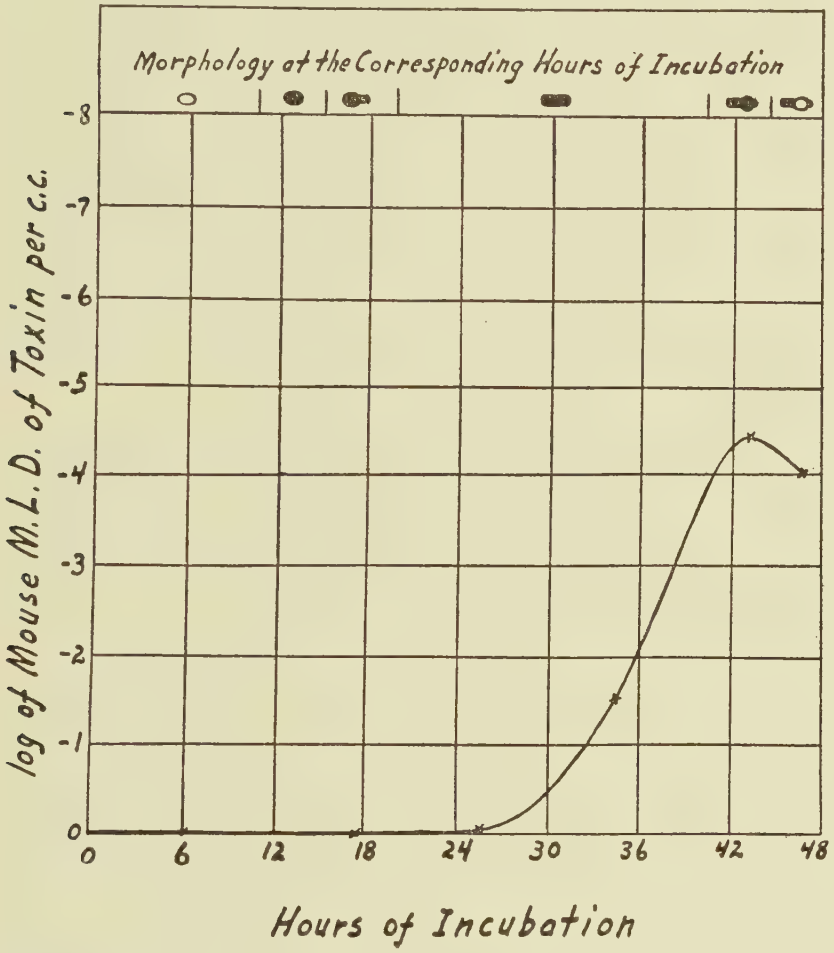

FIG. 8. AVERAGE TOXICITY OF THE CL. BOTULINUM CULTURES AT VARIOUS GROWTH PHASES

SUMMARY

With *Cl. botulinum*, and to a less definite degree with other organisms, the optimum hydrogen-ion concentration for sporula-

tion in a phosphate buffered medium is slightly acid and in a number of cases found to be at a pH of about 6.2 to 6.3.

Fermentable carbohydrates have an inhibiting effect on sporulation, mainly due to the production of acid and consequent high hydrogen-ion concentration produced. Whenever sufficient acid is produced to bring the pH below that at which growth can occur no sporulation takes place. With *Cl. botulinum* the limiting hydrogen-ion concentration for growth is about pH 6.

Increasing concentrations of inorganic salts will inhibit sporulation before inhibiting growth. In the case of *Cl. botulinum* the chlorides seem to be quite effective in inhibiting sporulation. Nitrates in concentration of .1 per cent have an inhibiting effect on the sporulation of *Cl. botulinum*. Ammonium salts have a stimulating effect on the sporulation of *Cl. botulinum*, as have also sulphates but to a lesser extent.

Certain elements seem to be more essential for sporulation than for growth. *Cl. botulinum* does not sporulate in a medium consisting of peptone only. Addition of various salts to peptone shows that ammonium and phosphate ions will induce sporulation while other ions will not. The calcium ion seems to have an added stimulating effect when added to ammonium and phosphate ions.

Increasing concentrations of oxygen inhibit sporulation before inhibiting growth. The optimum oxygen concentration for the sporulation of the anaerobes seems to be as close to 0 as it is possible to get by ordinary means. Neither growth nor sporulation was obtained with a number of anaerobes at an oxygen conconcentration of 2 cm. of mercury. Except with *Cl. tetani* and *Cl. novvi* some sporulation occurred at an oxygen tension of 1 cm. of mercury. In the case of the aerobes no sporulation was obtained at an oxygen tension of 2 cm. of mercury, although fair growth was observed.

A number of experiments were performed with *Cl. botulinum* to study the change of various properties as the organisms went through the spore cycle. The properties studied were: Morphology, cataphoretic potential, thermal resistance, oxygen sensitivity, pH of medium, and toxicity of medium. In each case the

change of the different properties of the organisms was correlated with the morphology.

The cataphoretic potential of spores was lower than that of the specific vegetative cells. The germinating spores had the highest potential.

Thermal death point determinations showed that a great number of the spores produced were not very resistant to high temperatures and must be regarded as failures. Only 1 to 2 per cent of the newly formed spores survived heating at 90°C. for a few minutes, 10 per cent survived 80°C., 20 per cent survived 70°C., and 50 per cent survived 60°C.

The experiment on oxygen sensitivity showed that only vegetative cells were oxygen sensitive. These were so sensitive that most of them were killed by less than two to three minutes exposure to air. The change from the nonsensitive spore to the exceedingly sensitive vegetative cell was so abrupt that only in a few cases was the sensitivity of the intermediary stages obtained.

Toxin production did not begin appreciably until the organisms were about to stop multiplying and sporulation began. During the period of sporulation the toxin titer increased very rapidly and reached a maximum at the time sporulation was complete. It then decreased due to the destruction of the toxin, especially if the toxin was allowed to come in contact with the air.

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DEVELOPMENT OF FLAGELLA ON GERMINATING SPORES[1]

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The only observation on the development of flagella on germinating spores which has come to my attention is that of Klein (1889). Klein used an organism he called *B. leptosporus* n.sp. (Klein). He made hanging drop preparations of the spores and observed them continuously at a temperature of about 35°C. The germination was generally complete in five and three-quarters hours. No motion of the organisms during germination was observed. Not until six hours and fifty minutes did characteristic motion begin to appear. At this time many four-membered chains of cells could be seen. From this observation it is impossible to tell exactly when and how flagella develop on the newly formed cell.

Working with a number of motile spore-forming bacteria including *B. vulgatus*, *B. cereus*, and *B. flavus* the author set about making flagella stains of the germinating spores. The stain used was that described by Leifson (1930). The method was essentially as follows: Week-old agar slant cultures, containing practically no vegetative cells, were suspended in distilled water and the suspension heated to 70°C. for ten minutes to kill any vegetative cells. A number of bouillon tubes were then heavily inoculated by means of a pipette with the heated suspension. At different intervals of time a tube was removed from the incu-

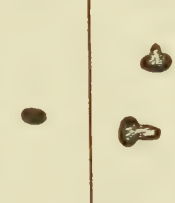
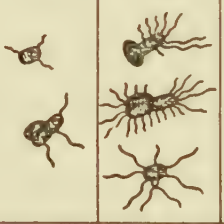
[1] Part of a dissertation submitted to the Department of Hygiene and Bacteriology of the University of Chicago to fulfil certain requirements for the degree of Doctor of Philosophy.

bator, a hanging drop made to determine motility and the organisms washed in distilled water. Flagella stains were made of

Table 1

Time and Manner of Development of Flagella on Newly Formed Vegetative Cells.

Motility and Morphology after Indicated Hours of Incubation

Organisms used		1¼ Hrs.	1½ Hrs.	1¾ Hrs.	2 Hrs.	2½ Hrs.
B.vulgatus	Motility	no motile forms	no motile forms	no motile forms	some motile forms	many motile forms
	Morphology					
B.cereus	Motility	no motile forms	some motile forms	-	sluggish motility	active motility
	Morphology					
B.flavus	Motility	no motile forms	some motile forms	many motile forms	good motility	good motility
	Morphology					

these washed organisms. The results with three of the organisms are given in table 1.

It is evident from table 1 that flagella begin to grow out of the organisms while they are still in the process of germination. The flagella do not grow out all at once but one by one. They evidently grow very rapidly; perhaps as much as 1 micron every two to three minutes. The organisms evidently become motile with only 2 or 3 short flagella present.

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